

1 **ELF3 polyQ variation in *Arabidopsis thaliana* reveals PIF4-independent role in**
2 **thermoreponsive flowering.**

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14 **Short title: ELF3/PIF4 independence in plant adult thermal responses**

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16

1 **ABSTRACT**

2 Plants have evolved elaborate mechanisms controlling developmental responses to
3 environmental stimuli. A particularly important stimulus is temperature. Previous work
4 has identified the interplay of PIF4 and ELF3 as a central circuit underlying thermal
5 responses in *Arabidopsis thaliana*. However, thermal responses vary widely among
6 strains, possibly offering mechanistic insights into the wiring of this circuit. ELF3
7 contains a polyglutamine (polyQ) tract that is crucial for ELF3 function and varies in
8 length across strains. Here, we use transgenic analysis to test the hypothesis that
9 natural polyQ variation in ELF3 is associated with the observed natural variation in
10 thermomorphogenesis. We found little evidence that the polyQ tract plays a specific role
11 in thermal responses beyond modulating general ELF3 function. Instead, we made the
12 serendipitous discovery that ELF3 plays a crucial, PIF4-independent role in
13 thermoreactive flowering under conditions more likely to reflect field conditions. We
14 present evidence that ELF3 acts through the photoperiodic pathway, pointing to a
15 previously unknown symmetry between low and high ambient temperature responses.
16 Moreover, in analyzing two strain backgrounds with vastly different thermal responses,
17 we demonstrate that responses may be shifted rather than fundamentally rewired
18 across strains. Our findings tie together disparate observations into a coherent
19 framework in which multiple pathways converge in accelerating flowering in response to
20 temperature, with some such pathways modulated by photoperiod.

21

22 **AUTHOR SUMMARY**

1 **Understanding plant responses to elevated temperature is crucial in a warming**
2 **world that threatens crop yields. Previous work suggested that the protein PIF4 is**
3 **a master regulator of early flowering at elevated temperatures in short days**
4 **typical of temperate cold seasons. However, short days are not usually paired**
5 **with elevated temperatures in the field. We show that the protein ELF3 is**
6 **essential for thermoresponsive early flowering in the more realistic scenario of**
7 **long days. We further demonstrate that this role is independent of PIF4. Our**
8 **study suggests that several pathways are important for thermoresponsive**
9 **flowering, with some (like PIF4) operating only under certain day lengths.**

10

11 **INTRODUCTION**

12 The responses of plants to temperature variation are of central importance to food
13 security in a changing world [1]. Therefore, the elucidation of the genetic pathways
14 underlying these responses has been a key mission of plant science [2]. Many previous
15 studies examined the phenomena of circadian temperature compensation [3–5],
16 thermoresponsive flowering [6–10], and temperature effects on plant morphology [11–
17 16]. Several have converged on PIF4 as a master regulator of temperature responses,
18 and ELF3 as an input to PIF4 integration, among many other genes and pathways
19 (REF). Given known regulatory interactions between ELF3 and PIF4 [17–19], it is
20 reasonable to predict that both operate in the same pathway for thermal response
21 phenotypes [20]. Recent reports focusing on one such phenotype, hypocotyl elongation,
22 support this expectation [14–16].

1 ELF3 serves to repress hypocotyl elongation by reducing PIF4 levels. This
2 repression of PIF4 occurs at both the transcriptional level, through the role of ELF3 in
3 the Evening Complex (EC) [17,19], and at the post-translational level, through PIF4
4 destabilization by phytochrome phyB [21]. Light sensing enforces circadian oscillations
5 of the EC and other components, leading to calibration of the circadian clock [22,23],
6 resulting in diurnal repression of hypocotyl elongation through repression of PIF4 and
7 PIF5 [17,19]. ELF3 also plays a crucial role as a flowering repressor [24]. Consequently,
8 *elf3* null mutants show elongated hypocotyls even in the light, and flower early.

9 PIF4 is one of a family of basic helix-loop-helix (bHLH) “phytochrome-interacting
10 factors” (PIFs), transcription factors with overlapping functions promoting
11 skotomorphogenesis. Under dark conditions, the PIFs act to target phyB for ubiquitin-
12 mediated degradation by the E3 ubiquitin ligase COP1, thereby repressing
13 photomorphogenesis [25]. Under light conditions, degradation of PIFs is mediated by
14 direct interactions with photoactivated phyB [21]. PIF4 is distinct from the other PIFs in
15 having specific roles in temperature sensing and flowering [26]. *pif4* null mutants show
16 short hypocotyls with photomorphogenic attributes even in the dark [27].

17 At elevated ambient temperatures (27°-29°) the wiring of these signaling
18 pathways changes. Several independent studies have recently found that elevated
19 temperatures, specifically during dark periods [28], inhibit the activity of the EC by an
20 unknown mechanism [14–16], leading to increased expression of *PIF4* and its targets
21 [11,26]. This increased PIF4 activity leads to several morphological temperature
22 responses through various signaling pathways [13,26]. *PIF4* is also required for the
23 acceleration of flowering at 27°C under short photoperiods [9,28], though these

1 observations have been disputed [29]. In contrast, under continuous light, *pif4* null
2 mutants have an intact temperature-dependent acceleration of flowering [11]. Lastly,
3 *pif4* null mutants lose the normal elongation of petioles under high temperatures [11]. It
4 is unclear why PIF4 does not affect thermoresponsive flowering under continuous light;
5 yet, this phenomenon may reflect low PIF4 levels under these conditions due to
6 inhibition by phyB. Under longer photoperiods and higher temperature a flowering
7 acceleration still exists [7,11], which suggests a PIF4-independent thermoresponsive
8 flowering pathway. Nonetheless, recent reviews of the literature tend to emphasize the
9 primacy of PIF4 in this response [10,30,31], although the condition of elevated
10 temperature with short photoperiods is probably rare in the field.

11 Recent studies have identified ELF3 as a plausible upstream regulator of PIF4 in
12 thermal responses [14–18]. However, others have implicated different candidates, such
13 as FCA [13], and mathematical modeling has suggested that ELF3/EC complex
14 regulation alone is insufficient to explain PIF4 thermal regulation [14,32]. The exact
15 mechanisms of this response have yet to be unraveled.

16 Specifically, the mechanism by which EC/ELF3 activity is reduced under elevated
17 temperatures (“temperature sensing”) is not known. We recently used transgenic
18 experiments to demonstrate that ELF3 function is dependent on the unit copy number of
19 its C-terminal polyglutamine (polyQ) tract [33]. This domain is likely disordered, and
20 disordered domains evince structural changes in response to physical parameters such
21 as temperature [34]. Thermal remodeling of this polyQ tract is a plausible mechanism by
22 which ELF3 activity could be modulated through temperature. This polyQ tract also
23 shows substantial natural variation [33], potentially serving as a factor underlying natural

1 variation in thermoresponsive phenotypes. For example, in flies, variable repeats are
2 associated with local temperature compensation adaptations [35]. In short, the ELF3-
3 polyQ is an attractive candidate for adaptive variation in the ecologically relevant trait of
4 temperature response [36].

5 In this study, we used transgenic polyQ variants of ELF3 in two *A. thaliana*
6 genetic backgrounds to dissect the contribution of the polyQ tract to temperature
7 response. We show that polyQ repeat copy number modulates temperature sensing by
8 affecting overall ELF3 function. Surprisingly, we found that ELF3's role in
9 thermoresponsive flowering appears to be entirely independent of PIF4. We postulate
10 that ELF3's primary role in thermoresponsive flowering is PIF4-independent and occurs
11 through the photoperiodic pathway, and that this role is in turn dependent on the genetic
12 background.

13

14 **RESULTS**

15 *The hypocotyl elongation temperature response is modulated by the ELF3 polyQ tract*
16 *affecting overall gene function.*

17 Many recent studies noted the involvement of ELF3 in temperature-dependent
18 hypocotyl elongation [14–16,37], concluding that ELF3 protein activity is reduced under
19 elevated temperatures, thereby relieving ELF3 repression of PIF4. PIF4 up-regulation
20 then leads to the observed hypocotyl elongation. We examined whether polyQ tract
21 variation in ELF3 in two backgrounds affects hypocotyl elongation at 27° (Fig. 1). We
22 previously showed that ELF3 polyQ variation has pleiotropic background-dependent
23 effects, with nonlinear associations between polyQ tract length and quantitative

1 phenotypes (including hypocotyl elongation at 22°C; ref. 33). Certain variants (16Q for
2 Ws, >20Q for Col) generally complemented *e/f3* null mutant phenotypes in Col and Ws
3 *A. thaliana* strains, whereas other variants complemented only specific phenotypes or
4 behaved as hypomorphs across all tested phenotypes. Here, we observed similar
5 trends for thermoresponsive hypocotyl elongation (Fig. 1). For example, in the Ws
6 background (Fig. 1A), the endogenous ELF3 variant (16Q) partially complements the
7 *e/f3* null mutant; another variant (9Q) fully complements the hypocotyl temperature
8 response. Other polyQ variants behaved as hypomorphs in Ws. In the Col background
9 (Fig. 1B), the endogenous 7Q variant, among other variants, failed to rescue the
10 response, agreeing with our previous observation that these transgenic lines are
11 hypomorphic in this background [33]. Deleting the entire polyQ tract eliminated
12 thermoresponsive hypocotyl elongation in both Col and Ws backgrounds. We next
13 addressed whether the observed phenotypic variation among polyQ variants was due to
14 variation in thermosensing or variation in general ELF3 function. We found that robust
15 thermal responses were strongly correlated with the overall functionality of each ELF3
16 variant in hypocotyl elongation (Fig. 1C), such that variants with intact thermal
17 responses exhibited short hypocotyls at 22°C, whereas ELF3 variants with defective
18 thermal responses exhibited elongated hypocotyls regardless of temperature. Together,
19 these results suggest that the ELF3 polyQ tract controls repression of hypocotyl
20 elongation regardless of temperature, rather than sensing temperature specifically.
21 Nonetheless, our transgenic ELF3 polyQ lines remain informative as an allelic series of
22 ELF3 function to understand the role of ELF3 in the de-repression of PIF4, which is
23 thought to underlie thermomorphogenesis [14–16,37–39].

1
2 **Fig. 1.** Response to elevated temperature (27°, relative to 22°) among transgenic lines
3 expressing ELF3-polyQ variants. Mean response and error were estimated by
4 regression, based on two independently-generated transgenic lines for each genotype,
5 with n >= 30 seedlings of each genotype in each condition (Table S1). WT = Ws, *elf3* =
6 *elf3* mutant+vector control, 0Q = *elf3* mutant+*ELF3* transgene lacking polyQ, etc. Error
7 bars indicate standard error of the mean. (A): Ws (Wassilewskija) strain background.
8 Lines are generated in an *elf3-4* background. (B): Response in the Col (Columbia) strain
9 background, lines were generated in an *elf3-200* background. (C): Temperature
10 response is a function of ELF3 functionality (repression of hypocotyl elongation at 22°).
11 Simple means of 22° hypocotyl length, regression estimates of temperature response.
12 PCC = Pearson correlation coefficient; p-value is from a Pearson correlation test.
13
14 *Expression of PIF4 and PIF4 targets as a function of temperature and ELF3.*
15 To evaluate the hypothesis that the thermal response defects in the transgenic lines
16 was due to up-regulation of PIF4 and PIF4 targets, we measured transcript levels of
17 *PIF4* and its target *AtHB2* in seedlings of selected lines from both backgrounds at 22°C
18 and 27°C (Fig. S1). Like others [15,16], we observed an inverse relationship between
19 ELF3 functionality and transcript levels of *PIF4* and *AtHB2*, with larger effects on *PIF4*
20 expression. The ELF3 lines with the strongest thermal response (e.g. 16Q in the Ws
21 background) showed the most robust de-repression of *PIF4* at elevated temperature.
22 However, *elf3* null mutants retained some *PIF4* up-regulation under these conditions,
23 especially in the Ws background. We conclude that ELF3-mediated de-repression of

1 PIF4 is involved in thermal responses as suggested by prior studies [15,16]; however,
2 de-repression of PIF4 and its targets may not be sufficient to explain the entirety of
3 thermal response defects in *elf3* null mutants.

4

5 *ELF3 polyQ variation affects thermoresponsive adult morphology and flowering time.*
6 Following the expectation that ELF3's thermal response acts through PIF4, we
7 reasoned that ELF3 should also play a role in other PIF4-dependent thermal responses.
8 One well-known response to elevated temperature is adult petiole elongation. *pif4*
9 mutants fail to show this response when grown at elevated temperatures [11]. We
10 measured petiole length in the ELF3 polyQ transgenic lines, expecting that, due to
11 general PIF4 de-repression, poorly-functioning ELF3 polyQ lines would show no
12 response (perhaps due to constitutively elongated petioles, similar to hypocotyls; Fig.
13 2). In stark contrast to this expectation, we found that all lines had a robust petiole
14 response to temperature (Fig. 2A, B). This effect was apparent in both Ws (Fig. 2A) and
15 Col backgrounds (Fig. 2B). Moreover, this response was actually accentuated in *elf3*
16 null mutants and in poorly-functioning ELF3 polyQ variants (Fig. 2A, B).

17

18 **Fig. 2.** Adult plant responses to elevated temperature (27°, relative to 22°) in long days
19 among transgenic lines expressing different ELF3-polyQ variants. (A) and (C):
20 Response in the Ws (Wassilewskija) strain background. Lines are in an *elf3-4*
21 background. (B) and (D): Response in the Col (Columbia) strain background, lines are
22 in an *elf3-200* background. (A) and (B) display PL:LL temperature response, (C) and (D)
23 display RLN temperature response. Average responses and errors were estimated in a

1 regression model accounting for variation between experiments (Table S2), based on
2 two to three independently-generated transgenic lines for each genotype. n >= 24 plants
3 overall for each genotype in each condition. PL:LL = petiole to leaf length ratio at 25
4 days post germination, RLN = rosette leaf number at flowering, WT = wild type, *elf3* =
5 *elf3* mutant+vector control, 0Q = *elf3* mutant+*ELF3* transgene with entire polyglutamine
6 removed, etc. Error bars indicate standard error.

7

8 Further, we measured flowering time in transgenic lines as the number of rosette
9 leaves at flowering (Fig. 2C, D). PIF4 is not required for the accelerated flowering
10 temperature response under longer photoperiods [11]. Hence, we expected that loss of
11 *ELF3* function should also not affect thermoresponsive flowering. In contrast to this
12 expectation, in the Col background, *elf3* mutants had an abrogated flowering response
13 to elevated temperature (Fig. 2D). Moreover, most variants in the Col background
14 entirely failed to rescue this phenotype.

15 Unlike Col, Ws is known to lack a robust flowering response to elevated
16 temperature under these conditions [40], and indeed, variants in the Ws background
17 generally showed no thermoresponsive flowering (Fig. 2C). Thus, ELF3 polyQ variation
18 does not suffice to enhance the negligible thermoresponsive flowering in the Ws
19 background under these conditions. In light of this data, the roles of ELF3 and PIF4 in
20 the elevated temperature response appear to be independent of one another under
21 these experimental conditions and for these traits. These results are intriguing, given
22 that the PIF4 pathway is the best-recognized mechanism for thermoresponsive

1 flowering at high temperatures [9,10,30,31]. Therefore, we suggest that ELF3 acts in a
2 PIF4-independent pathway for thermoresponsive flowering at high temperatures.

3

4 *ELF3 regulates thermoresponsive flowering under long days, and is not required for*
5 *PIF4-dependent thermoresponsive adult morphologies.*

6 We directly addressed the relationship of ELF3 and PIF4 in adult thermoresponsive
7 phenotypes by growing *pif4* and *elf3* mutants with various thermal treatments. Previous
8 experiments with *pif4* mutants used different conditions from ours, specifically a later
9 transfer to elevated temperature [11]. Hence, it was possible that the observed
10 inconsistencies between *elf3* and *pif4* effects on adult thermoresponsive phenotypes
11 were a trivial consequence of experimental conditions. Specifically, the effects of
12 elevated temperature during the early seedling stages (the conditions we use) may
13 induce pathways irrelevant to treatments at later, vegetative stages. Thus, we tested
14 both transfer conditions under long days (Fig. 3). We found that the effect of different
15 experimental conditions is negligible, though the earlier 27°C treatment showed a
16 slightly stronger morphological response (Fig. 3A, B). Thus, the timing of the 27°C
17 treatment (early seedling vs. vegetative stage) does not substantially affect adult
18 thermoresponsive traits. Further, our results under long days were similar to previous
19 observations under continuous light [11], showing that PIF4 is essential for petiole
20 elongation (Fig. 3B), but dispensable for thermoresponsive flowering (Fig. 3C). Our
21 PIF4 results were in direct contrast to ELF3, which was dispensable for petiole
22 elongation (Fig. 3B), but essential for thermoresponsive flowering (Fig. 3C). These

1 results confirm the apparent independence of ELF3 and PIF4 in these specific
2 responses.

3

4 **Fig. 3.** *elf3* and *pif4* null mutant phenotypes are independent under LD treatments and
5 robust to conditions. (A), (B), and (C): 22°: constant 22° LD growth; 27° 14d: transfer
6 from 22° to 27° at 14 days post-germination; 27° 1d: transfer from 22° to 27° at 1 day
7 post-germination. (A): Col (WT), *elf3-200*, and *pif4-2* plants grown under long days with
8 three different temperature regimes were photographed at 20 days post germination.
9 Experiment was repeated with similar results. (B and D): Petiole elongation responses
10 of the indicated genotypes, measured by ratio of petiole to whole leaf length at 25 days
11 post germination. Regression analysis of data in Table S3.

12

13 One open question was whether the dispensability of ELF3 for petiole elongation
14 reflected increased importance of other inputs to PIF4, such as FCA, which is involved
15 in PIF4-dependent thermoresponsive petiole elongation in 7-day-old seedlings [13]. We
16 therefore measured adult thermoresponsive petiole elongation in *fca* mutants (Fig.
17 S2A), and unexpectedly found no substantial difference between *fca* mutants and WT
18 Col. Regulatory rewiring across development may remove FCA and ELF3 as inputs to
19 PIF4-dependent thermomorphogenesis in 25-day-old adult plants.

20 A second question was whether loss of *ELF3* function can affect
21 thermoresponsive flowering in the Ws strain under other temperature conditions. We
22 therefore assayed flowering in Ws and the Ws null mutant *elf3-4* at 16°C and 22°C (Fig.
23 S2B). Under these conditions, Ws robustly accelerated flowering at 22°C, whereas *elf3-*

1 4 showed no perceptible difference in flowering between the two temperatures. Thus,
2 ELF3's role in thermoresponsive flowering is not restricted to the Col strain or a certain
3 temperature, but rather is necessary for whatever thermoresponsive reaction norm a
4 strain may have for flowering.

5

6 *ELF3 and PIF4 regulate adult thermoresponsive phenotypes independently.*
7 If ELF3 and PIF4 were truly independent in controlling thermal responses of adult
8 phenotypes under long days, then *elf3 pif4* double mutants would show approximately
9 additive phenotypes. We generated *elf3 pif4* double mutants and subjected them to the
10 same experiments as above. Our results indicated that flowering and petiole elongation
11 constitute independent temperature responses, with PIF4 controlling the former and
12 ELF3 controlling the latter in additive fashions (Fig. 4). That is, *elf3 pif4* double mutants
13 showed negligible thermoresponsive flowering like *elf3*, and a negligible petiole
14 response like *pif4*. Additionally, *elf3 pif4* flowered slightly later than *elf3* at 22°, while
15 maintaining a negligible thermal response in flowering, indicating that *elf3* mutants are
16 not simply restricted by a physiological limit of early flowering. The additivity of these
17 phenotypes establishes that, under these conditions, ELF3 and PIF4 must operate in
18 separate thermal response pathways.

19

20 **Fig. 4.** Double mutant analysis confirms PIF4 and ELF3 independence in adult
21 temperature responses and non-redundancy of PIF4 with PIF5. (A): Col, *elf3-200, pif4-*
22 *2, and elf3-200 pif4-2* plants grown under long days with two different temperature
23 regimes were photographed at 25 days post germination. (B): Petiole elongation

1 responses of the indicated genotypes, measured by ratio of petiole to whole leaf length
2 at 25 days post germination. (C): Flowering temperature response of indicated
3 genotypes, measured by rosette leaf number (RLN) at flowering. (B) and (C): n > 8
4 plants for each genotype in each treatment. All “27°” plants were seeded and incubated
5 one day at 22° before transfer to 27°. Experiments were repeated with similar results.
6 Regression analysis of data reported in Tables S6 and S7.

7

8 Previous studies have indicated that other members of the PIF family, such as
9 PIF1, PIF3, and PIF5, have minimal roles in these same thermal response phenotypes
10 [11,26,41]. *pif4 pif5* double mutants show slightly abrogated thermoresponsive flowering
11 even under 12 hour light : 12 hour dark photoperiods [28]. These previous findings
12 suggest that our results are not explained by redundancy between PIFs. However, to
13 further exclude this possibility, we evaluated thermoresponsive flowering in *pif4 pif5*
14 mutants (Fig. 4D), because PIF5 is most often considered to act redundantly with PIF4
15 [28,42,43]. As expected, both *pif5* single mutants and *pif4 pif5* double mutants
16 demonstrate intact thermoresponsive flowering. These observations indicate that
17 redundancy with other PIFs is not responsible for the apparent independence of PIF4
18 and ELF3.

19 Overall, the strong photoperiod-dependence of PIF4-related thermoresponsive
20 flowering necessitates the existence of some pathway or pathways independent of PIF4
21 under long days, given the persistence of the phenomenon under these conditions.
22 Based on our data, ELF3 acts in one such pathway.

23

1 *Thermoresponsive flowering under long days can operate through the photoperiodic*
2 *pathway.*

3 ELF3 operates in thermoresponsive flowering at low ambient temperatures via the
4 photoperiodic pathway, through repressing *GI* expression, after which *GI* in turn directly
5 activates FT [44,45]. To evaluate whether this pathway might explain our results, we
6 measured transcript levels of *GI* and *CO* in wild-type and *elf3* mutants under 22°C and
7 27°C (Fig. 5A). We found that *GI* is strongly up-regulated in *elf3* null mutants of Col and
8 Ws backgrounds, confirming previous reports in Col [37,45]. Further, wild-type Ws
9 showed approximately five-fold higher basal *GI* levels compared to Col, which did not
10 increase at higher temperatures. In contrast, Col showed very low basal *GI* levels that
11 increased at higher temperatures to approximately the same levels as Ws. *CO* levels,
12 however, were not substantially increased by either *elf3* mutation or increased
13 temperature, consistent with previous reports [8,45]. Thus, robust thermoresponsive
14 flowering was correlated with low basal levels of *GI*, and with temperature-dependent *GI*
15 up-regulation, as observed in Col. High basal *GI* levels in Ws may be associated with
16 other thermoresponsive deficiencies at high temperatures in this strain [40,46,47].
17 These observations support the model under which ELF3 acts in the photoperiodic
18 pathway to engender thermoresponsive flowering, just as it does in response to lower
19 ambient temperatures [8,45].

20

21 **Fig. 5.** ELF3 and *GI* regulate thermoresponsive flowering. (A): Temperature-responsive
22 expression of photoperiodic pathway components. Expression of each gene is
23 quantified relative to levels in Ws at 22° (Ws 22 = 1.0). This experiment was repeated

1 with similar results. *elf3-4*: *elf3* null in Ws background; *elf3-200*: *elf3* null in Col
2 background. (B): Thermoresponsive flowering in various flowering mutants. LD RLN =
3 rosette leaf number at flowering under long days. * : interaction term for genotype by
4 environment at $p < 0.01$; details of regression model in Table S8. (C) Thermoresponsive
5 petiole elongation in various flowering mutants. For (B) and (C), $n \geq 8$ plants of each
6 genotype in each condition; white boxes indicate measurements at 22° , red boxes
7 indicate measurements at 27° . *gi*: *gi-2*, *co*: *co-101*, *spy*: *spy-3*, *soc1*: *soc1* T-DNA
8 insertion, *elf3*: *elf3-200*. This experiment was repeated with similar results. (D): Models
9 of thermoresponsive flowering under long and short photoperiods. Dashed edges
10 indicate speculated temperature sensing mechanisms. Edges with increased weight
11 indicate relative increases of influence between conditions. Pathways are indicated,
12 along with other important actors reported elsewhere.

13

14 If the photoperiodic pathway contributes to thermoresponsive flowering at
15 elevated ambient temperatures in long days (LD), we would expect mutants in this
16 pathway to show abrogated thermal responses, as they do under short days (SD), along
17 with members of the autonomous pathway [7]. These two pathways also contribute
18 independently to thermoresponsive flowering at low temperatures (16°C vs. 23°C) [6,8].
19 Altogether, we would expect that a photoperiodic thermoresponsive flowering pathway
20 would operate independently of both PIF4 and the autonomous pathways in long days.
21 It is not clear whether the autonomous pathway would be independent of PIF4, given
22 known interactions between FCA and PIF4 [13].

1 To evaluate whether these past results under other conditions also apply to long
2 days and elevated temperatures, we measured flowering time at 22°C and 27°C in
3 mutants in the photoperiodic pathway (*gi*, *co*, Fig. 5B). We also tested mutants of the
4 gibberellin pathway (*spy*), and a terminal floral integrator (*soc1*), which are not expected
5 to be necessary for thermoresponsive flowering. We found robust thermal responses in
6 all mutants except *elf3* and *gi*, similar to previous results under different conditions
7 [7,8,44,45]. These results implicate GI (but not CO) as an actor in thermoresponsive
8 flowering at elevated temperatures. Collectively, these experiments suggest that the
9 photoperiod pathway is necessary in promoting thermoresponsive flowering in long
10 days, and expression data in this and other studies suggests that ELF3 is likely to act
11 within this pathway.

12

13 **DISCUSSION**

14 ELF3 and PIF4 are both crucial integrators of temperature and light signaling in
15 controlling *A. thaliana* development. Recent literature has emphasized the centrality of
16 PIF4-dependent thermoresponsive regulation in a variety of phenotypes, including in
17 flowering [9,10,30]. Here, we show that PIF4 is dispensable for thermoresponsive
18 flowering under long photoperiod conditions [11], and that ELF3 is essential for
19 thermoresponsive flowering under these conditions. Our results integrate previous
20 knowledge about thermoresponsive flowering, and identify at least one pathway for this
21 response that does not involve PIF4. Moreover, we show that while polyQ variation in
22 ELF3 affects ELF3 function, the polyQ tract is unlikely a temperature-responsive
23 component in itself. Our results allow us to integrate the many disparate findings of

1 current studies into classic models of thermal responses in *A. thaliana*, allowing a
2 comprehensive view of the genetic underpinnings of this agronomically crucial plant
3 trait.

4

5 *ELF3 polyglutamine variation appears to affect thermoresponsive traits by modulating*
6 *overall ELF3 activity.*

7 In previous work, we demonstrated that polyQ variation in ELF3 is (i) common, (ii)
8 affects many known ELF3-dependent phenotypes, and (iii) is dependent on the genetic
9 background [33]. Following the recent discoveries that ELF3 is involved with thermal
10 response [14–16], we confirmed that ELF3 polyQ variation also affects thermal
11 response phenotypes in a background-dependent fashion. However, we found little
12 support for the hypothesis that the polyQ tract has a special role in temperature
13 sensing. Instead, as was the case for other ELF3-dependent phenotypes, ELF3 polyQ
14 variation appeared to affect overall ELF3 functionality, with less functional ELF3 variants
15 lacking robust temperature responses. However, a more exhaustive series of polyQ
16 variants may be required for revealing polyQ-specific effects, in particular because the
17 molecular mechanism(s) by which polyQ variation affects ELF3 functionality remain
18 unknown.

19

20 *ELF3-PIF4 relationship in thermomorphogenesis.*

21 One question that remains unanswered is to what extent ELF3 participates in PIF4-
22 dependent thermoresponsive morphologies. While our study and previous work
23 [14,16,37] support a PIF4-ELF3 link in thermoresponsive hypocotyl elongation, this

1 relationship disappears in the analogous case of thermoresponsive petiole elongation.
2 These results can be explained by many hypotheses. For instance, it is possible that
3 ELF3 regulation of PIF4 is only relevant at the early seedling stage. Another possible
4 hypothesis is that ELF3 regulation of PIF4 in some instances is sufficient but not
5 necessary for thermal responses. More studies are needed to understand the
6 mechanistic details of the ELF3 and PIF4 relationship in thermomorphogenesis.

7

8 *Natural variation in temperature response.*

9 Several studies have found that different *A. thaliana* strains respond to temperature
10 differently, either shifting or inverting the reaction norm of the phenotype in question
11 [40,46,47]. Ws has a shifted reaction norm with respect to temperature compared to Col
12 for photoperiod-related phenotypes, including flowering. For instance, Ws displays
13 accelerated flowering at 23°C vs. 16°C [40], but accelerates flowering no further at
14 27°C. Here, we show that this acceleration requires ELF3, like the elevated temperature
15 acceleration in Col. Another example of differential mutational effects among strains is
16 that *gi* mutants in the Ler background display robust thermoresponsive flowering [6,7]. It
17 is unclear whether this finding is due to altered wiring of pathways between these
18 backgrounds.

19

20 *Thermoresponsive flowering requires either PIF4 or ELF3, depending on photoperiod.*

21 Under various conditions, both ELF3 and PIF4 have been found to be crucial for
22 thermoresponsive flowering. Other members of the autonomous and the photoperiodic
23 pathways have also been implicated in thermoresponsive flowering [6–8] (besides other

1 pathways, [48]). Consequently, some combination of these pathways, modulated by
2 experimental conditions, must require ELF3 and/or PIF4. We and others [11,28] have
3 observed that PIF4 and its paralogs are not required for proper thermoresponsive
4 flowering under longer photoperiods. Furthermore, we and others [8,45] have shown
5 that ELF3 and the photoperiod pathway (excluding CO) are essential for proper
6 thermoresponsive flowering under long days. It has been previously shown that PIF4
7 and the photoperiodic pathway contribute to thermoresponsive flowering via
8 independent pathways [9], suggesting that under longer photoperiods PIF4 activity is
9 inhibited, allowing other mechanisms to dominate thermoresponsive flowering.

10 We propose a model of thermoresponsive flowering, in which PIF4, ELF3, the
11 photoperiodic pathway, and other pathways interact depending upon condition and
12 genetic background (Fig. 5D). Under short days or other short photoperiods, phyB
13 activity is down-regulated, leading to up-regulation of PIF4 [21,49–51], which at high
14 levels occupies the promoter of the flowering integrator *FT* and induces flowering [9].
15 However, under longer photoperiods, phyB up-regulation leads to an attenuation of
16 PIF4 activity, and consequently the role of PIF4 and other PIFs becomes negligible [11].
17 This allows canonical ambient temperature responses (such as the photoperiodic
18 pathway, including ELF3, [8,45]) to take a dominant role in thermoresponsive flowering.
19 Constitutive overexpression of PIF4, PIF5, and PIF3 under long day conditions induces
20 early flowering [29], supporting the hypothesis that differences in PIF levels underlie the
21 photoperiod-dependence of PIF4's role. Several reports have indicated that GI and
22 COP1, but not CO, are involved in thermoresponsive flowering [7,8,45], with GI directly
23 binding the *FT* promoter [45]. Under each of these conditions, *FT*-induced flowering is

1 activated by a different signaling cascade. This interpretation leads to a coherent view
2 of how light and temperature responses are integrated in this important plant trait.

3 To summarize, at least three independent mechanisms have been described that
4 promote thermoresponsive flowering in any context. These include the photoperiodic
5 pathway (PHYB/ELF3/GI/COP1), the autonomous pathway
6 (PHYA/FCA/FVE/TFL1/FLC), and the PIF4-dependent pathway
7 (PIF4/H2A.Z/gibberellin), all of which converge by regulating *FT* (although the last
8 pathway may also act through other integrators [28,29]). The collective results of our
9 experiments and previous work suggest that the first two pathways are necessary but
10 not sufficient for thermoresponsive flowering, and that the third (PIF4) is sufficient but
11 not necessary for thermoresponsive flowering. Further study will be necessary in
12 understanding the interdependencies of the three pathways. For instance, it has been
13 suggested that PIF4 binding to the *FT* promoter is dependent on cooperativity with a
14 second photoperiod-controlled actor [32].

15 In conclusion, we observe that ELF3 is involved in the hypocotyl response to
16 elevated temperature as reported previously, and that this response can be abrogated
17 by poorly-functioning ELF3 polyQ variants. We further demonstrate that ELF3 has little
18 effect on the petiole temperature response, and is necessary for the flowering
19 temperature response, suggesting that it functions independently of PIF4, potentially in
20 the photoperiodic pathway. These results reiterate the complexity of these crucial
21 environmental responses in plants, and will serve as a basis for further development of
22 our understanding of how plants respond to elevated temperatures. In the context of

1 climatic changes, this understanding will serve those attempting to secure the global
2 food supply.

3

4 **MATERIAL AND METHODS**

5 *Plant materials and growth conditions.* All mutant lines (except *pif4-2 elf3-200*) were
6 either described previously or obtained as T-DNA insertions from the Arabidopsis
7 Biological Resources Center at Ohio State University [52,53], and are described in
8 Table S10. *pif4-2 elf3-200* was obtained via crossing and genotyping. T-DNA insertions
9 were confirmed with primers described in Table S9. For hypocotyl assays, seedlings
10 were grown for 15d in incubators set to SD on vertical plates as described previously
11 [33]. All plates were incubated at 22° for one day, after which one replicate arm was
12 transferred to an incubator set to 27°, with another replicate arm maintained at 22°. For
13 flowering time assays, plants were stratified 3-5d at 4° in 0.1% agarose and seeded into
14 Sunshine #4 soil in 36-pot or 72-pot flats to germinate at 22° under LD. Replicate arms
15 were subsequently transferred to 27° LD conditions as indicated, with others remaining
16 at 22°. Different temperature treatments of the same experiment were identical with
17 respect to randomization, setup, and format. At 25d, petiole length and whole leaf length
18 (including petiole) of the third leaf were measured, and the ratio of these values was
19 further analyzed. Flowering was defined as an inflorescence ≥1cm tall; at this point,
20 date and rosette leaf number were recorded.

21

22 *Trait data analysis.* All data analysis was performed using R v3.2.1 [54]. Where
23 indicated, temperature responses were modeled using multiple regression in the form
24 $\text{Phenotype} \sim \mu + \beta_G \text{Genotype} + \beta_T \text{Temperature} + \beta_{GxT} (\text{Genotype} \times \text{Temperature}) +$
25 $\beta_E \text{Experiment} + \text{Error}$. All experiments were included in models for transgenic
26 experiments, and thus the β_E term describes systematic variation between experiments,
27 whereas line-specific effects among transgenics should be modeled in the error term.
28 Where temperature responses are reported, they consist of the $\beta_T + \beta_{GxT}$ terms and
29 associated errors ($\sqrt{\sigma_T^2 + \sigma_{GxT}^2}$ where σ_T is the standard error for β_T and σ_{GxT} is the
30 standard error for β_{GxT}), and thus are corrected for systematic experimental variation

1 and temperature-independent genotype effects. Analysis scripts and data are provided
2 at <https://figshare.com/s/129525f02ef6e66f7bed>.

3
4 *Gene expression analyses.* Seedlings were grown for 1d under LD at 22°, after which
5 one replicate arm was transferred to LD at 27°, with another replicate arm remaining at
6 22°, and all seedlings were harvested 6d later at indicated times. At harvest, ~30mg
7 aerial tissue of pooled seedlings was frozen immediately in liquid nitrogen and stored at
8 -80°. RNA extraction, cDNA synthesis, and real-time quantitative PCR were performed
9 as described previously [33], using primers in Table S9. Transcript levels were
10 quantified using the $\Delta\Delta C_t$ method [55].

11

12

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19

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- 26
- 27

1

2 **Supporting Information Captions**

3 **Fig. S1. Expression analysis of PIF4 and AtHB2 depends on temperature, genetic**
4 **background, and ELF3 functionality.** Error bars represent the standard error of the
5 mean across 3 technical replicates. White bars represent 22° expression, red bars 27°
6 expression for each line. Tissue was collected from 7d seedlings at ZT0. This
7 experiment was repeated with similar results.

8

9 **Fig. S2. Regulation of adult thermoresponsive traits by ELF3 and FCA is**
10 **independent of PIF4 and modulated by genetic background.** Flowering temperature
11 response of indicated genotypes under indicated conditions, measured by rosette leaf
12 number (RLN) at flowering. For each experiment, n > 10 plants for each genotype in
13 each treatment. Regression analysis of data in Tables S4 and S5.

14

15 **Table S1. Regression analysis of hypocotyl elongation temperature response**
16 **among Col and Ws transgenic lines.**

17 **Table S2. Regression analysis of petiole : leaf length ratio and rosette leaf**
18 **number at flowering temperature response among Col and Ws transgenic lines.**

19 **Table S3. Regression analysis of rosette leaf number at flowering and petiole :**
20 **leaf length ratio temperature responses in elf3 and pif4.**

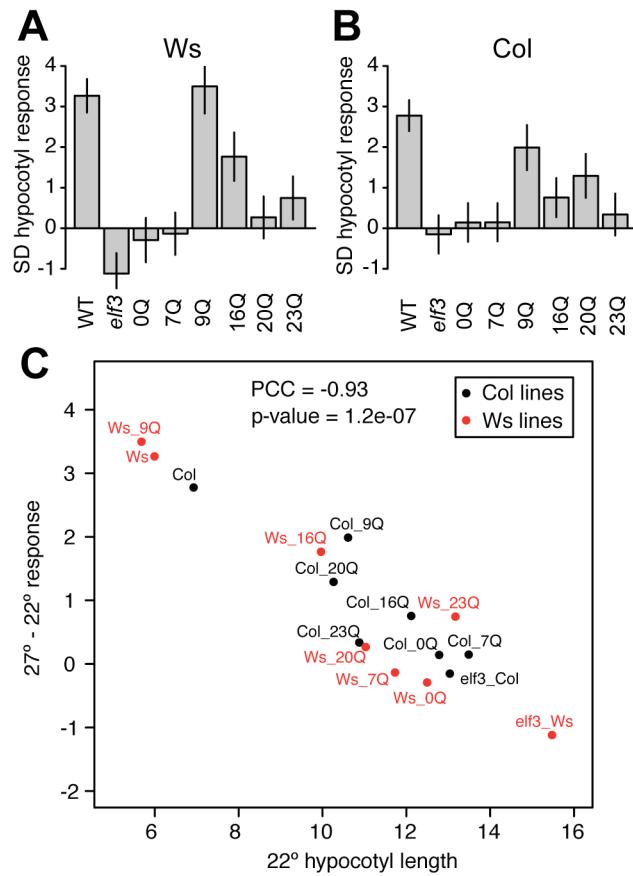
21 **Table S4. Regression analysis of rosette leaf number at flowering temperature**
22 **response in Ws and elf3-4.**

- 1 **Table S5.** Regression analysis of petiole : leaf length ratio temperature response
2 in *Col* and *fca* mutants.
- 3 **Table S6.** Regression analysis of rosette leaf number at flowering temperature
4 response in *elf3 pif4* double mutants.
- 5 **Table S7.** Regression analysis of rosette leaf number at flowering temperature
6 response in *pif4 pif5* double mutants.
- 7 **Table S8.** Regression analysis of rosette leaf number at flowering and petiole :
8 leaf length ratio temperature responses in flowering pathway mutants.
- 9 **Table S9.** Primers used in this study.
- 10 **Table S10.** Mutant lines used in this study.

11

1

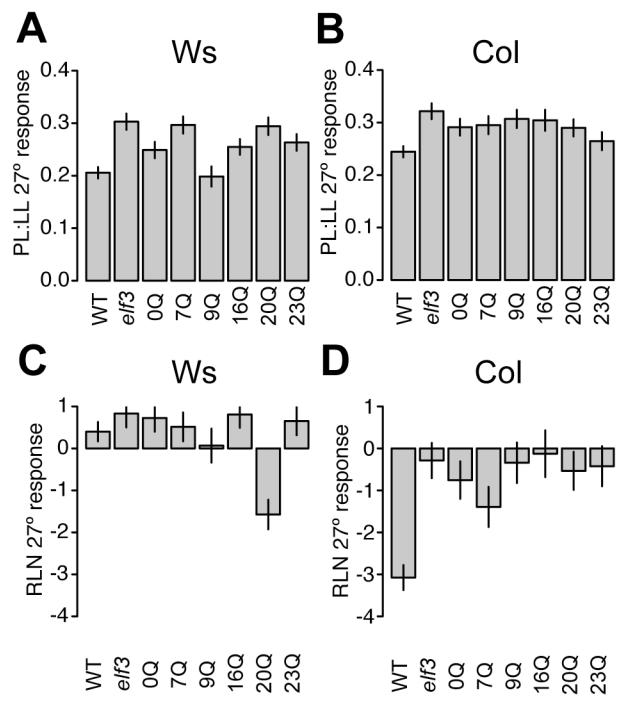
Figure 1



2

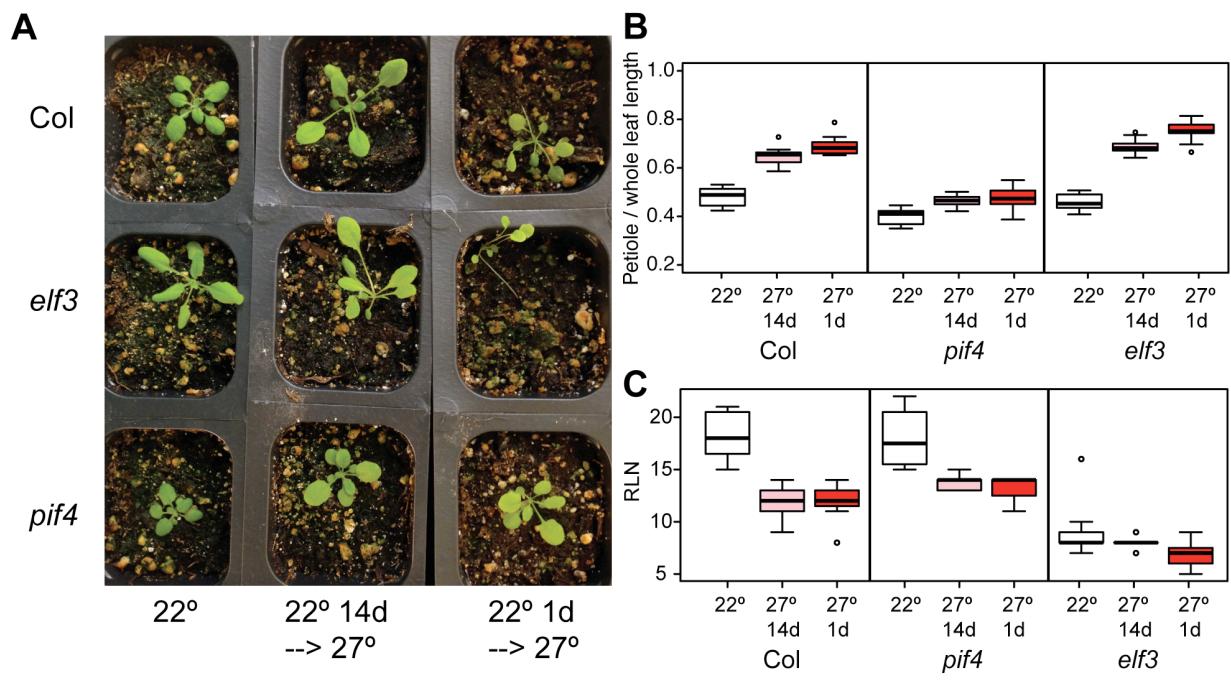
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Figure 2



1

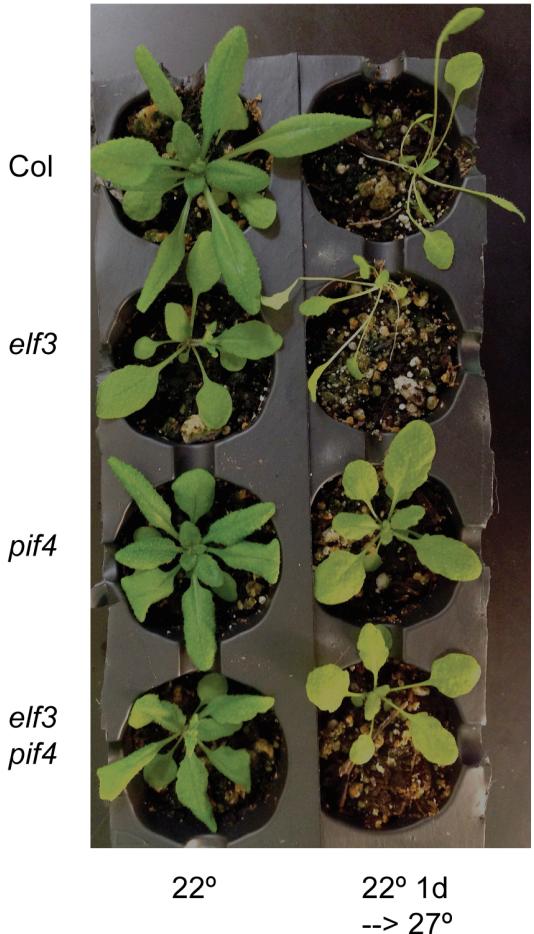
Figure 3



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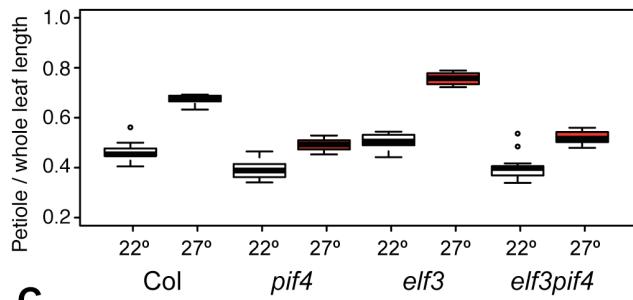
Figure 4

A

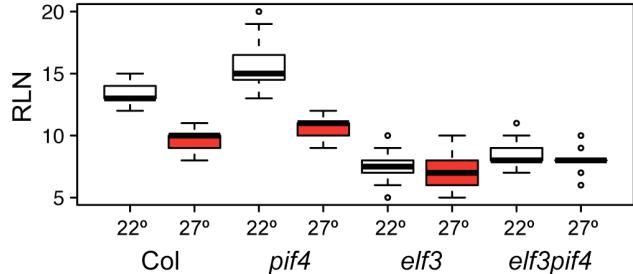


1

B



C



D

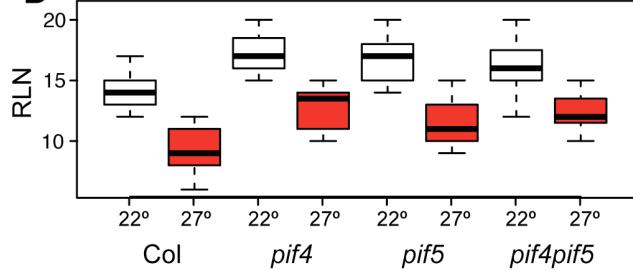
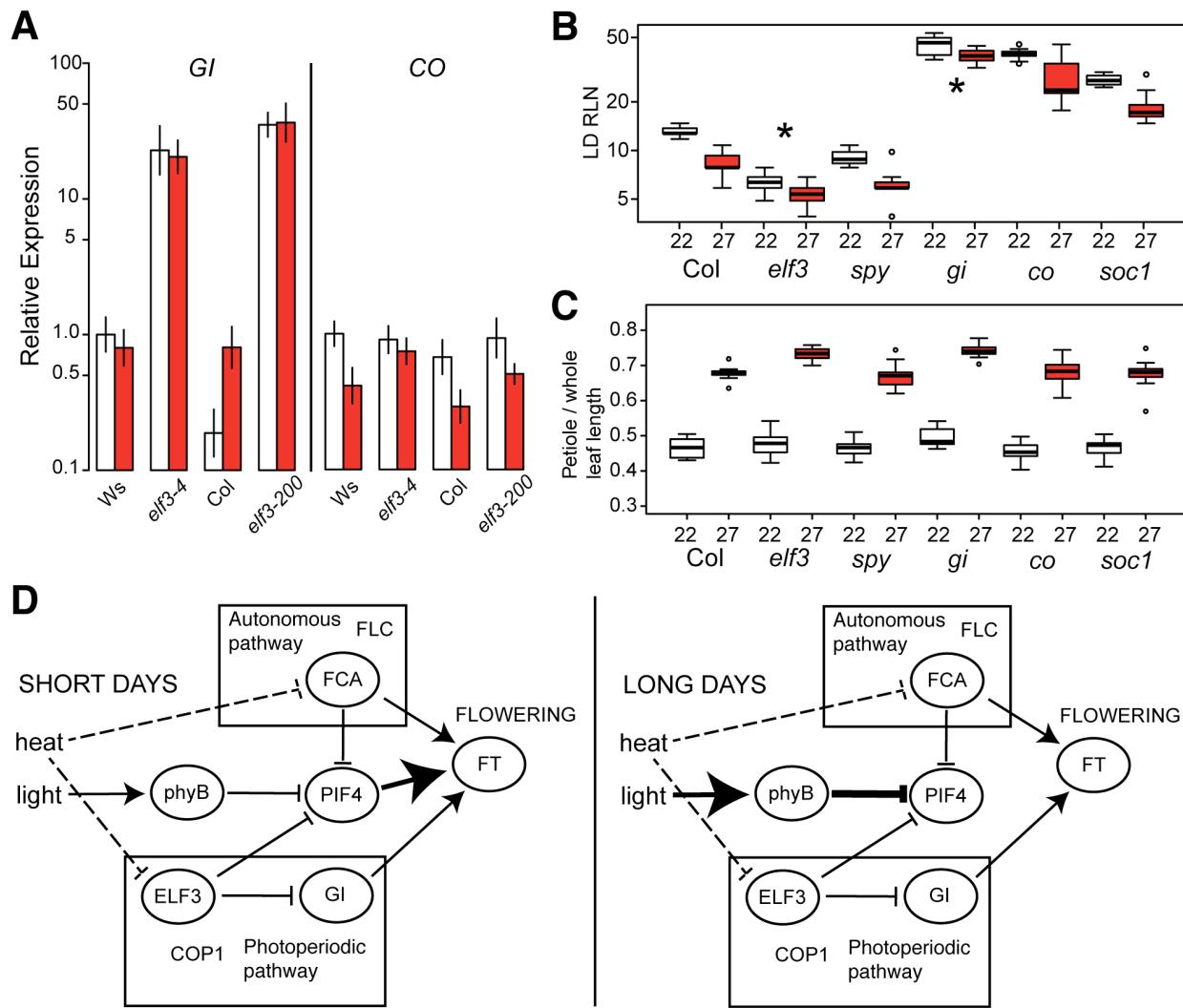


Figure 5



1